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13. ABSTRACT (Maximum 200 Words)

We hypothesize that chromosomal loss of the Y chromosome in prostate cancer is a specific tumorigenic event. Transfer of the Y chromosome by microcell mediated chromosome transfer into PC-3 cells resulted in suppression of tumor growth. However, these PC-3 hybrids containing the Y chromosome did grow in soft agar indicating a lack of correlation between *in vitro* and *in vivo* tumor growth. From hybrid cells with fragments of the Y chromosome we concluded suppression of tumorigenesis correlates with either the short arm of the Y or two small regions on the q arm. The data from the functional assay was correlated with data from deletions in patient material. Using a complete genomic microarray specific for the Y chromosome that we constructed from BAC clones, we performed array CGH (Comparative genomic hybridization) of prostate tumor samples. These studies indicate there is a small region near on the short arm of the Y chromosome near the centromere that is deleted in 50% of tumor cells. The region of functional suppression and the region of deletion coincide indicating that Y chromosome loss is a significant step in prostate cancer.

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Prostate cancer, y chromosome, tumor suppressor gene

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INTRODUCTION:

Loss of the Y chromosome has been noted in prostate cancer by other investigators (Konig et al., 1996; Arps et al., 1993; Arps et al., 1993; Baretton et al., 1994; Jordan et al., 2001). These experiments did not resolve whether a specific region of the Y chromosome was deleted. Chromosomal loss is a hallmark of a tumor suppressor gene. However, functional proof for a tumor suppressor gene had not previously been done. We transferred an intact Y chromosome into the prostate cancer cell line PC-3. Introduction of the Y chromosome resulted in the suppression of tumor growth in nude mice. To obtain a finer resolution for loss of the Y chromosome, we constructed an array of BAC clones for the Y chromosome. Array comparative genomic hybridization was used to determine the smallest region of loss. Both the functional study and the deletion study indicate that a region on the short near the centromere of the Y chromosome is important in the development of prostate cancer.

BODY:

Task 1. PCR analysis of tumor samples, Months 1-15

a. Isolate DNA from tumor samples

Over 300 patient samples have been collected and entered into the repository. We have isolated DNA from frozen tissue and more recently from paraffin blocks.

b. Continue to develop Y chromosome specific markers

We have continued to develop Y chromosome specific markers. Since we have defined a region that is deleted in prostate tumors we are developing markers (both PCR and quantitative PCR) to analyze tumors.

c. Analyze tumors with Y specific markers
AND

d. Quantitate the frequency of Y chromosome change

We have analyzed 50 tumors with primers developed from the commonly deleted region identified by array CGH (see below). We find that 45% of the tumors are deleted for the markers. To determine whether this was a tumor specific phenomenom, we also tested DNA from unaffected seminal vesicles in one case. This normal tissue was also deleted for this specific region.

We then tested whether deletion of this region of the Y chromosome was found in normal (blood) tissue from both individuals with prostate cancer and those that have a normal DRE and a PSA value of <2.5 ng/ml. We found for the marker MA59S1 that 6.5% of men with tumors have a deletion while only 3.2% of the controls have this loss.

Task 2. In situ hybridization of interphase tumor cells, Months 3-15

a. Develop Y chromosome specific probes for in situ hybridization

Since we have used the BAC array to define the smallest region of deletion on the Y chromosome, we now have BAC probes for this region. The clones RP11 441G8 is at the center of the deletion and is being used for hybridization. We also developed control clones that are never deleted (312H22). A sample of the hybridization of these markers is given in Figure 1.

b. Analyze tumors tissue samples with Y specific markers

We have initiated the hybridization of the BAC clones to paraffin sections from tumors. These studies are currently being completed.

Task 3. Genomic DNA array, Months 1-15

a. Develop Y chromosome specific clones for DNA arrays

The Y chromosome specific BAC array was developed from the BAC clones we had identified from the Y chromosome as well as the tiling path that was used in the sequencing of the Y chromosome. These clones gave us coverage of 22.8 Mb of the euchromatic DNA on the Y chromosome. Although the Y chromosome is considered to be 58.4 Mb, the rest of the sequence is repetitive DNA. Each clone from the path was subcloned and tested with PCR primers specific for the BAC. A summary of all the data we have generated for these clones is listed in Table 1.

b. Establish conditions for stamping arrays and hybridization

After spending a great deal of time trying to optimize conditions for producing arrays, we enlisted the help of Spectral Genomics in Houston. Although we received the arrays and were able to obtain some data, the company was having difficulty with changes in the genomic arrays upon storage. They were able to solve this problem and are now printing a whole new set of arrays for us (free of charge).

c. Hybridize tumor samples to DNA array

Despite the suboptimal quality of the arrays, we were able to hybridize tumor DNA to our genomic arrays (see figure 2). As can be seen, there is a specific region of the short arm of the Y chromosome that is consistently deleted (figure 2 and table 2).

d. Analyze data from arrays

According to the array results, deletions in the region of the BAC 441G8 are seen in half of the 18 samples that could be definitively analyzed. These are very exciting data since a specific region of the Y that is lost quite frequently has been identified. Also noted on the table is that these changes were seen in frozen samples as well as paraffin embedded tumors (See N1). In addition no deletions were seen in 5 normal individuals.

Task 4. Microcell transfer of Y chromosome fragments, Months 4-18

a. Transfer the Y chromosome fragments into PC-3 cells

We have transferred a Y chromosome marked with the hisD selectable marker into PC-3 cells. Please see accompanying manuscript for details. During the process of microcell transfer, we were able to identify clones that had deletions of the Y chromosome. By analyzing these clones carefully, we were able to limit the region of the Y chromosome that suppressed tumor growth of PC-3/

We also found that although the introduction of the Y chromosome into PC-3 cells resulted in loss of growth in nude mice, the microcell hybrids were able to grow on soft agar. To further test the cells, we isolated clones of PC-3 hybrids that had grown in soft agar. We tested 9 of these subclones in nude mice. One of the nine clones formed tumors in nude mice. By analyzing markers on these clones we were able to determine the smallest region of the Y chromosome involved in tumor suppression.

In response to reviewers of our manuscript, we did another experiment to insure that the introduction of the selectable marker was not the reason for tumor suppression. We transfected PC-3 cells with the vector pHTtkm3 and selected with histidinol-containing medium. All the animals injected with these clones formed tumors in nude mice comparable to PC-3 cells.

We spend quite a bit of time attempting to make directed deletions using a cre-lox strategy. We were able to target the vectors into the chromosome. However, we were unsuccessful in creating a deletion. Consequently we have since concentrated on the BAC arrays and human tumors. The mapping with this strategy is even finer than what we could have obtained using directed deletions.

b. Isolate independent clones and assay for Y specific markers

c. Perform in vitro assays for tumor growth

d. Inject tumors into nude mice and quantitate tumor growth

e. If necessary, develop metastasis assays

We have completed these studies and have submitted them for publication (attached).

Task 5. Candidate gene identification, Months 18-24

a. Determine the smallest region for candidate gene from all data

The smallest region of this deletion is 750 Kb.

b. Identify the clones involved in the region

Three BAC clones span this area: 441G8, 344D2, 370N2, and 71C1. In these clones are the TSPY gene cluster and the gene RMBY. None of the other potential transcripts have open reading frames.

c. Analyze the genomic sequence for ESTs and potential genes

This region has been thoroughly dissected and there are only two potential candidates.

d. Isolate cDNAs corresponding to these genes

We have made PCR and realtime PCR primers for these genes to determine: 1. the rate of loss of genomic sequences in tumor samples and 2. the expression levels of these genes.

e. Test cDNAs in functional assay

The TSPY gene cluster is quite complex. We will start with transfection of whole BAC clones to determine which region is the correct one. We will then identify the key gene.

Task 6. Prepare manuscripts and final report, Months 20-24

We have submitted the manuscript on suppression of tumor formation of PC-3 cells by the Y chromosome (see appendix). We have provisional acceptance from Genes, Chromosomes and Cancer after revision.

Our array studies are nearly ready to publish. We are waiting for reprinted arrays to do a final hybridization for the paper. We are in the process of confirming the deletions and testing for frequency by paraffin in situ hybridization and by quantitative real time PCR. My graduate student will be submitting this manuscript within the next month.

KEY RESEARCH ACCOMPLISHMENTS:

- In situ hybridization of the HisD gene confirmed the location of the selectable marker at the end of the p arm of the Y chromosome.
- Introduction of the Y chromosome into PC-3 prostate cancer cells results in the suppression of tumor growth in nude mice.
- Introduction of the Y chromosome into PC-3 prostate cancer cells does not result in the suppression growth in soft agar.
- In38 microcell PC-3 hybrids identified with fragments of the Y chromosome were characterized with markers.
- Subclones (28) that grow on soft agar were isolated from three of the PC-3 hybrid clones.
- Nine soft agar derived clones were injected into nude mice and only one of the subclones grew in nude mice.
- The region of the Y chromosome associated with the suppression of tumor growth has been limited to the short arm and two small regions of the Y chromosome.
- The genomic array of BAC clones representing the Y chromosome have been isolated and verified by PCR.
- The genomic array of BAC clones have been printed on slides by Spectral Genomics.
- DNA isolated from frozen tumors and paraffin embedded tumors has been hybridized to the BAC arrays.
- In half the tumors (9/18) a small deletion of the short arm of the Y chromosome is seen.
- The smallest region of deletion is 750 Kb and has the TSPY cluster and the RBMY gene.
- FISH and quantitative PCR probes have been made to test for the frequency of this deletion in prostate cancer.

REPORTABLE OUTCOMES:

- Abstract presented at the Cold Spring Harbor Meeting on Cancer Genetics and Tumor Suppressor Genes in August 2002, by S. Vijayakumar, M. Bannerjee, D.K. Garcia, T. Bracht, J. Kagan, and S.L. Naylor
- Graduate student presentation of the data at the Texas Genetic Society Meeting in April 2002 and in April 2004.
- A manuscript: The human Y chromosome suppresses the tumorigenicity of PC-3, a human prostate cancer cell line, in athymic nude mice, by Sapna Vijayakumar, Dawn Garcia, Chuck H. Hensel, Mohua Banerjee, Todd Bracht, RuiHua Xiang, Jacob Kagan

and Susan L. Naylor, has been revised and sent for publication in Genes Chromosomes and Cancer.

- The Y chromosome BAC array has been constructed and is open to the community.
- The Y BAC clones have been verified and are available to the community.
- The Y chromosome array analysis is being prepared for publication.

CONCLUSIONS:

We have shown that the introduction of the Y chromosome into PC-3 cells results in the suppression of tumor growth in nude mice. Surprisingly the insertion of the Y chromosome does not inhibit growth in soft agar. The analysis of clones with fragments of the Y chromosome was compared to chromosomal deletions seen in human prostate cancer tumors. Array CGH (comparative genomic hybridization) indicate that there is a region of 750 Kb that is lost in 50% of the tumors. This region coincides with the region necessary for tumor suppression in the PC-3 line.

Reference List

Arps,S., Rodewald,A., Schmalenberger,B., Carl,P., Bressel,M., and Kastendieck,H. (1993). Cytogenetic survey of 32 cancers of the prostate. Cancer Genet. Cytogenet. *66*, 93-99.

Baretton,G.B., Valina,C., Vogt,T., Schneiderbanger,K., Diebold,J., and Lohrs,U. (1994). Interphase cytogenetic analysis of prostatic carcinomas by use of nonisotopic in situ hybridization. Cancer Res. *54*, 4472-4480.

Jordan,J.J., Hanlon,A.L., Al Saleem,T.I., Greenberg,R.E., and Tricoli,J.V. (2001). Loss of the short arm of the Y chromosome in human prostate carcinoma. Cancer Genet. Cytogenet. *124*, 122-126.

Konig,J.J., Teubel,W., Romijn,J.C., Schroder,F.H., and Hagemeijer,A. (1996). Gain and loss of chromosomes 1, 7, 8, 10, 18, and Y in 46 prostate cancers. Hum. Pathol. *27*, 720-727.

APPENDICES; Figure 1. In situ hybridization of BACs from the Y chromosome deletion.
Figure 2. Analysis of the Y BAC array for the tumor N12.
Table 1. Clones for the Y chromosome.
Table 2. Results from the array CGH analysis of the Y chromosome.

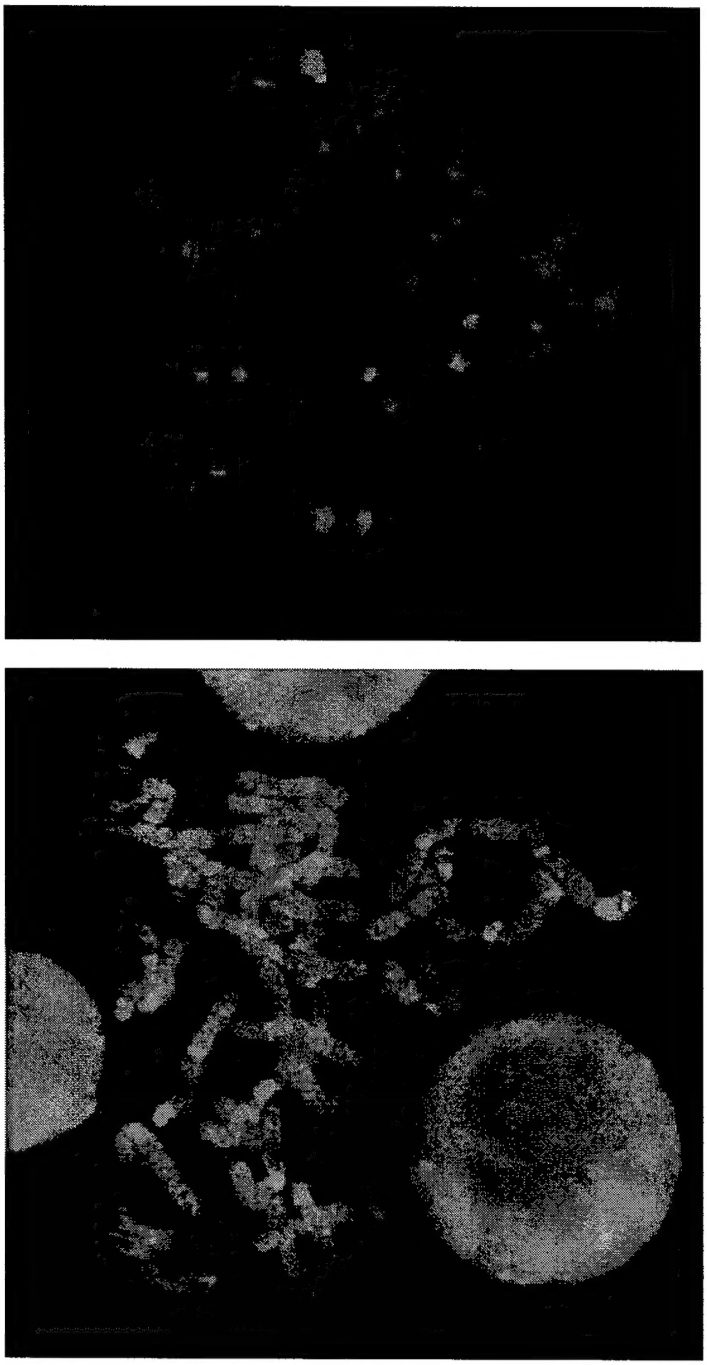

Figure 1. Hybridization of Y specific probes to chromosomes from a normal human male. Top, the probe is 441G8, the region that is deleted in prostate cancer samples. Bottom is a control probe 312H22 located on the long arm of the Y chromosome.

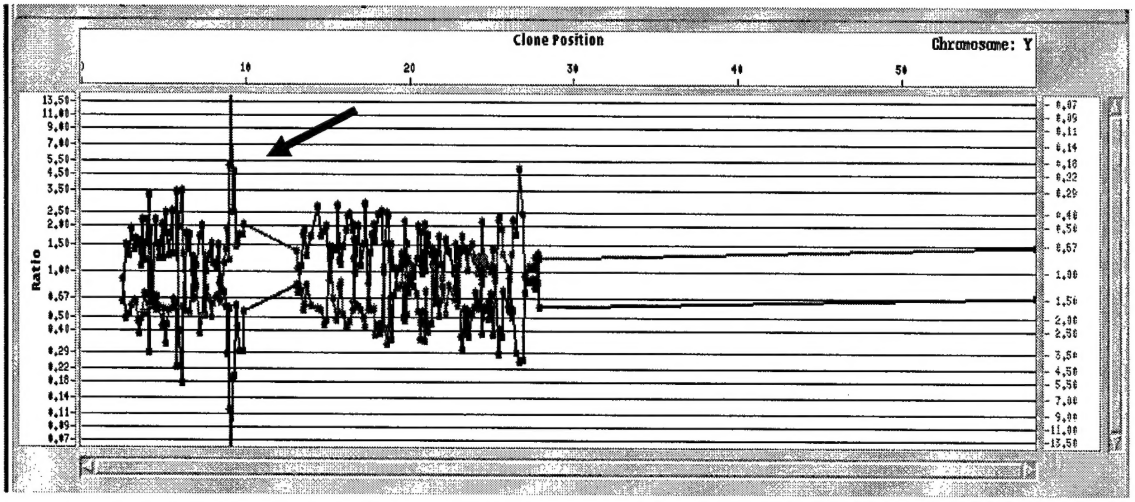

Figure 2. Deletion seen in prostate tumors. Shown here is a ratio plot of the hybridization of tumor DNA to the Y chromosome BAC array. The peak located at the arrow is seen in 45% of samples. Hybridization is done labeling the tumor with Cy3 and the normal male control with Cy5 and vice versa. The upward deflection of the peak in red accompanied by the downward defection of the blue indicates a deletion of Y chromosome DNA.

Table 1: PCR Verification for Y Chromosome BAC Clones

	Accession No.	BAC Clone	Marker Tested	Result	DNA
2.5 Mb to Yp telomere					
	AC006040.3	RP11-400O10	SRY	POSITIVE	YES
	AC074181.1	RP11-515L2	515L2*	NEGATIVE	NO
	AC006157.2	RP11-414C23	ZFY	POSITIVE	YES
	AC006032.2	RP11-115E20	DXYS106	POSITIVE	YES
	AC006152.3	RP11-4N7	DYS 395	POSITIVE	YES
	AC011305.2	RP11-390E9	sY 721	POSITIVE	YES
	AC009479.4	RP11-278L6	sY 870	POSITIVE	YES
	AC019058.4	RP11-125B15	sY 872	POSITIVE	YES
	AC024038.6	RP11-349O6	AF20109	POSITIVE	YES
	AC012078.3	RP11-539O22	539O22*	POSITIVE	YES
	AC010094.5	RP11-336O5	sY 703	POSITIVE	YES
	AC010737.4	RP11-439L24	DXYS112	POSITIVE	YES
	AC010084.3	RP11-145J12	DYS 253	POSITIVE	YES
	AC010905.3	RP11-560B8	560B8*	POSITIVE	YES
	AC010106.2	RP11-575J5	575j5*	POSITIVE	YES
	AC024703.5	RP11-51N20	51N20*	POSITIVE	YES
	AC012077.4	RP11-524G14	sY 876	POSITIVE	YES
	AC010142.4	RP11-240N18	sY 875	POSITIVE	YES
	AC019060.5	RP11-125K5	sY 1008	POSITIVE	YES
	AC023423.5	RP11-430C23	sY 936	POSITIVE	YES
	AC010722.2	RP11-122L9	sY 2138	POSITIVE	YES
	AC010685.3	RP11-465A8	DYS 255	POSITIVE	YES
	AC010129.3	RP11-59N9	sY 2141	POSITIVE	YES
	AC012067.2	RP11-192N14	sY 2146	POSITIVE	YES
	AC012667.2	RP11-357C22	sY 716	POSITIVE	YES
	AC010081.4	RP11-65E7	DYS 256	POSITIVE	YES
	AC010874.3	RP11-118K2	118K2*	POSITIVE	YES
	AC010977.4	RP11-362J16	sY 2171	POSITIVE	YES
	AC016681.2	RP11-62H15	sY 866	POSITIVE	YES
	AC010140.3	RP11-218E11	sY 1011	POSITIVE	YES
	AC006335.2	RP11-492C2	DYS 379	POSITIVE	YES
	AC010154.3	RP11-573O23	DYS 257	POSITIVE	YES
	AC010144.4	RP11-309M4	sY 1091	POSITIVE	YES
	AC010728.4	RP11-258E22	258E22*	POSITIVE	YES
	AC013412.3	RP11-507A3	507A3*	POSITIVE	YES
	AC011297.3	RP11-115H13	DYS 266	POSITIVE	YES
	AC012068.5	RP11-196J6	sY 2234	POSITIVE	YES
	AC010104.3	RP11-540C18	DXS7855	POSITIVE	YES
	AC010143.3	RP11-301O17	sY 887	POSITIVE	YES

	AC007284.4	RP11-558K21	558k21*	POSITIVE	YES
	AC007247.5	RP11-305H21	DYS 261	POSITIVE	YES
	AC007274.3	RP11-105L10	DYS 260	POSITIVE	YES
	AC007275.4	RP11-109F19	DYS 288	POSITIVE	YES
	AC010678.4	RP11-108F14	DYS 54	POSITIVE	YES
	AC010902.4	RP11-549J7	549J7*	NEGATIVE	
	AC016749.4	RP11-504E20	SHGC-107423	POSITIVE	YES
	AC051663.9	RP11-475P15	sY 1103	POSITIVE	YES
	AC025731.12	RP11-48H21	48H21*	POSITIVE	YES
	AC016991.5	RP11-17E15	17E15*	POSITIVE	YES
	AC064829.6	RP11-375P13	sY 953	POSITIVE	YES
	AC009491.3	RP11-418M8	DYS 231	POSITIVE	YES
	AC007967.3	RP11-373F14	SHGC-80640	POSITIVE	YES
	AC068719.3	RP11-403P11	sY 894	POSITIVE	YES
50 kb gap	AC079126.3	CTB-45E23	REPEATS		
	AC079125.4	RP11-1188O8	DYS 392	POSITIVE	YES
	AC009952.4	RP11-175I4	DYS 258	POSITIVE	YES
	AC025732.9	RP11-116J19	116J19*	POSITIVE	YES
	AC006158.6	RP11-441G8	sY 1079	POSITIVE	YES
	AC006156.5	RP11-344D2	DYS 398	POSITIVE	YES
	AC025819.7	RP11-370N2	370N2*	POSITIVE	YES
	AC017019.3	RP11-182H20	DYS 379	POSITIVE	YES
	AC010891.2	RP11-453C1	453C1*	POSITIVE	YES
	AC006986.3	RP11-155J5	DYS 268	POSITIVE	YES
	AC006987.2	RP11-160K17	DYS 269	POSITIVE	YES
	AC010970.3	RP11-108I14	sY 2267	POSITIVE	YES
	AC069323.5	RP11-1126J10	1126J10*	NOT WORKING	
CENTROMERE - 3.05 Mb gap					
	AC011293.5	RP11-75F5	DYS 270	POSITIVE	YES
	AC012502.3	RP11-461H6	461H6*	POSITIVE	YES
	AC011302.3	RP11-333E9	DYS 271	POSITIVE	YES
	AC013735.5	RP11-558M10	558M10*	POSITIVE	NO
	AC004772.2	CTB-144J1	144j1*	POSITIVE	YES
	AC005942.2	CTC-298B15	298b15*	POSITIVE	YES
	AC002992.1	203M13	RP11 LIBRARY-NEGATIVE		
	AC004617.2	264M20	RP11 LIBRARY-NEGATIVE		

AC004810.1	CTB-69H8	69h8*	POSITIVE	YES
AC002531.1	486O2	RP11 LIBRARY-NEGATIVE		
AC004474.1	475I1	RP11 LIBRARY-NEGATIVE		
AC006565.4	CTC-484O7	484o7*	POSITIVE	YES
AC005820.1	CTC-494G17	494g17*	NEGATIVE	
AC010877.3	RP11-218F6	sY 2366	POSITIVE	YES
AC006376.2	RP11-386L3	DYS 276	POSITIVE	YES
AC007004.3	RP11-521D3	521D3*	NEGATIVE	
AC006383.2	RP11-498H20	sY 2375	POSITIVE	YES
AC006371.2	RP11-304C24	DYS 277	POSITIVE	YES
AC006370.2	RP11-292P9	DYS 246	POSITIVE	YES
AC018677.3	RP11-264A13	sY 2395	POSITIVE	YES
AC010720.4	RP11-53K10	sY 2384	POSITIVE	YES
AC010723.3	RP11-139C10	DYS 227	POSITIVE	YES
AC019191.4	RP11-312H22	312H22*	POSITIVE	YES
AC010726.4	RP11-224C16	DYS 280	POSITIVE	YES
AC010979.3	RP11-384N21	sY 882	POSITIVE	YES
AC010879.2	RP11-235I1	sY 2386	POSITIVE	YES
AC017032.3	RP11-292E8	sY 910	POSITIVE	YES
AC006989.3	RP11-225B4	SHGC-83159	POSITIVE	YES
AC011289.4	RP11-59K8	DYS 390	POSITIVE	YES
AC010972.3	RP11-133D3	sY 863	POSITIVE	YES
AC007007.3	RP11-551F5	sY 2478	POSITIVE	YES
AC006998.3	RP11-458M9	DYS 282	POSITIVE	YES
AC006382.3	RP11-494J4	DYS 281	POSITIVE	YES
AC006462.3	RP11-389B19	sY 2458	POSITIVE	YES
AC006336.4	RP11-508K5	sY 770	POSITIVE	YES
AC016671.3	RP11-12J24	REPEATS		
AC017020.4	RP11-185K15	SHGC-60455	POSITIVE	YES
AC011749.2	RP11-455E3	SHGC-78944	POSITIVE	YES
AC053516.10	RP11-442J5	sY 2544	POSITIVE	YES
AC010135.3	RP11-128D13	DYS 200	POSITIVE	YES
AC010128.3	RP11-15H4	15h4*	POSITIVE	YES
AC011751.2	RP11-478I15	DYS 289	POSITIVE	YES
AC016678.4	RP11-55O11	DYS 243	POSITIVE	YES
AC015979.4	RP11-538M13	DYS 200	POSITIVE	YES
AC007034.4	RP11-99M1	SHGC-5485	POSITIVE	YES
AC007043.3	RP11-507E21	sY 2545	POSITIVE	YES

AC006999.2	RP11-462A19	DYS 201	POSITIVE	YES
AC007042.3	RP11-399H17	sY 2568	POSITIVE	YES
AC091329.3	RP11-568H21	REPEATS		
AC007972.4	RP11-537C24	DYS 202	POSITIVE	YES
AC015978.4	RP11-529I21	DYS 241	POSITIVE	YES
AC068704.4	RP11-434F12	DYS 203	POSITIVE	YES
AC007742.4	RP11-357E16	DYS 211	POSITIVE	YES
AC095381.1	GAP1623			
AC009976.4	RP11-509B6	DYS 241	POSITIVE	YES
AC095380.1	GAP1622			
AC024183.4	RP11-268K13	268k13	POSITIVE	YES
AC007241.3	RP11-157F24	DYS 203	POSITIVE	YES
AC069130.6	RP11-468D10	DYS 241	POSITIVE	YES
AC073962.5	RP11-945E12	945E12*	POSITIVE	YES
AC068541.7	RP11-243P9	DYS 211	POSITIVE	YES
AC022486.4	RP11-569J3	DYS 208	POSITIVE	YES
AC007379.2	RP11-143C1	DYS 208	POSITIVE	YES
AC009235.4	RP11-392F24	DYS 212	POSITIVE	YES
AC007244.2	RP11-207L19	DYS 213	POSITIVE	YES
AC021210.4	RP11-389F23	sY 919	POSITIVE	YES
AC010133.4	RP11-118E9	sY 916	POSITIVE	YES
AC012062.4	RP11-80E19	sY 2608	POSITIVE	YES
AC010137.3	RP11-169D1	DYS 214	POSITIVE	YES
AC009977.4	RP11-576C2	sY 2615	POSITIVE	YES
AC010889.3	RP11-424G14	sY 971	POSITIVE	YES
AC010151.3	RP11-508P10	sY 969	POSITIVE	YES
AC009233.3	RP11-356K22	DYS 217	POSITIVE	YES
AC079157.3	RP11-1285C3	1285C3*	POSITIVE	YES
AC079261.2	RP11-1325K3	REPEATS		
AC079156.3	RP11-943F15	sY 1155	POSITIVE	YES
AC024250.6	RP11-684N2	sY 1155	POSITIVE	YES
AC009240.6	RP11-489O13	489O13*	POSITIVE	YES
AC011745.4	RP11-329C15	DYS 392	POSITIVE	YES
AC007678.3	RP11-256K9	DYS 219	POSITIVE	YES
AC009494.2	RP11-450B24	450B24*	POSITIVE	YES
AC026061.8	RP11-223K9	223K9*	NEGATIVE	NO
AC009489.3	RP11-339J4	sY 1013	POSITIVE	YES
AC007876.2	RP11-65G9	DYS 221	POSITIVE	YES
AC009239.3	RP11-470K20	470K20*	NEGATIVE	
AC010086.4	RP11-209I11	209i11	POSITIVE	YES
AC010141.2	RP11-220O2	DYS 225	POSITIVE	YES
AC021107.3	RP11-178M5	DYS 258	POSITIVE	YES
AC078938.3	CTC-480L15	480I15*	NEGATIVE	NO
AC024236.5	RP11-400I17	DYS 230	POSITIVE	YES

50 Kb gap

AC007322.4	RP11-553C13	DYS 400	POSITIVE	YES
AC007359.3	RP11-66M18	DYS 379	POSITIVE	YES
AC023342.3	RP11-95B23	DYS 77	POSITIVE	YES
AC025227.6	RP11-109G18	DYS 227	POSITIVE	YES
AC007320.3	RP11-477B5	DYS 77	POSITIVE	YES
AC008175.2	RP11-427G18	SHGC-7605	POSITIVE	YES
AC016694.2	RP11-123G1	123G1*	POSITIVE	YES
AC010080.2	RP11-5C5	sY 990	POSITIVE	YES
AC016911.6	RP11-473E1	473E1*	POSITIVE	YES
AC006366.3	RP11-86G22	DYS 235	POSITIVE	YES
AC010088.3	RP11-289L7	sY 2716	POSITIVE	YES
AC053490.2	RP11-140H23	DYS 236	POSITIVE	YES
AC007039.6	RP11-263A15	263A15*	POSITIVE	YES
AC006983.4	RP11-70G12	SHGC-1348	POSITIVE	YES
AC009947.2	RP11-39P20	DYS 12	POSITIVE	YES
AC016707.2	RP11-221K4	221K4*	POSITIVE	YES
AC016752.2	RP11-506M9	SHGC-9458	POSITIVE	YES
AC025246.6	RP11-589P14	SHGC103584	NEGATIVE	
AC073649.3	RP11-823D8	823d8	NEGATIVE	
AC073893.4	RP11-978G18	sY 707	POSITIVE	YES
AC068601.8	RP11-1067I16	sY 710	POSITIVE	YES
AC023274.2	RP11-307L15	307L15*	POSITIVE	YES
AC012005.4	RP11-533E23	SHGC-104362	POSITIVE	YES
AC013465.4	RP11-424J12	424J12*	POSITIVE	YES
AC016698.3	RP11-160O2	DYS 235	POSITIVE	YES
AC010153.3	RP11-535I13	REPEATS		
AC025735.4	RP11-214M24	sY 2716	POSITIVE	YES
AC010089.4	RP11-290O3	sY 579	POSITIVE	YES
AC006982.3	RP11-26D12	SHGC-35663	POSITIVE	YES
AC006338.5	RP11-539D10	DYS 236	POSITIVE	YES
AC016728.4	RP11-363G6	DYS 235	POSITIVE	YES
AC006386.4	RP11-566H16	DYS 237	POSITIVE	YES
AC006328.5	RP11-102O5	sY 2729	POSITIVE	YES
AC007562.4	RP11-497C14	sY 710	POSITIVE	YES
AC010682.2	RP11-251M8	sY 707	POSITIVE	YES
AC017005.6	RP11-100J21	DYS 241	POSITIVE	YES
AC007965.3	RP11-245K4	SHGC-9458	POSITIVE	YES
AC006991.3	RP11-270H4	SHGC-1348	POSITIVE	YES
AC024067.4	RP11-487K20	DYS 247	POSITIVE	YES
AC013734.4	RP11-557B9	DYS 247	POSITIVE	YES
AC019099.6	RP11-428D10	sY 1072	POSITIVE	YES
AC073880.5	RP11-1136L22	REPEATS		

30 Mb gap of heterochromatic repeat					
	AC068123.5	RP11-242E13	REPEATS		
20 kb gap					
	AC025226.4	RP11-57J19	SHGC-7991	POSITIVE	YES

800 kb to end of Yq telomere

* Custom-made primers

TOTAL	**198**	**DNA ISOLATED**	**177**
EXCLUDED	**21**		
FINAL	**177**		

Table 2. Hybridization of the BAC Array to Tumor Samples

Tumor Code	AGE	Gleason's	Stage	441G8-344D2 Y chromosome
N1 (Frozen)	57	7	T3BNOMX	No deletion
N1 (PET)	"	"	"	No deletion
N2 (Frozen)	65	8	T3ANOMX	No deletion
N3 (Frozen)	66	9	T3BNOM0	(Yq deleted)
N4 (Frozen)	66	7	T3BN0MX	No deletion
N5 (Frozen)	64	7	T2BN0MX	No deletion
N6 (Frozen)				Total Y deletion
N7 (Frozen)				Total Y gain
N8 (PET)*	63	7	T3BN0MX	?#
N10 (PET)	66	6	T2CNXMX	Deletion
N12 (PET)	64	6	T2CNXMX	Deletion
N15 (PET)			T2CNXMX	Deletion
n16 (PET)	56	7	II	Deletion
N18 (PET)	62	6	T2BNXMX	Deletion
N19 (PET)	75	5	T2 NXMX	Deletion
N20 (PET)	71	9	T3BN0MX	Deletion
N22 (PET)	59	7	T2CN0MX	Deletion
N23 (PET)	58	10	T3BN0MX	Deletion
N29 (PET)	63	9	T3BNOMX	?#
N50 (5u Frozen section)	62	8	T2CNXMX II	No deletion
888888 tumor DNA				No deletion
Total deletions				9 out of 18 (50%)

?# The pattern shows some amount of deletion. However, these were scored as no deletion.

The human Y chromosome suppresses the tumorigenicity of PC-3, a human prostate cancer cell line, in athymic nude mice.

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The loss of the Y chromosome is a frequent numerical chromosomal abnormality observed in human prostate cancer. In cancer, loss of specific genetic material frequently accompanies simultaneous inactivation of tumor suppressor genes (TSGs). It is not known whether the Y chromosome harbors such genes. To address the role of genes on the Y chromosome in human prostate cancer, we transferred a tagged Y chromosome into PC-3, a human prostate cancer cell line lacking a Y chromosome. A human Y chromosome was tagged with the *hisD* gene and transferred to PC-3 by microcell mediated chromosome transfer. Tumorigenicity of these PC-3 hybrids was tested *in vivo* and *in vitro* and the results were compared to the PCR analyses conducted on the PC-3 hybrids using Y chromosome specific markers. Out of 60 mice injected with 12 different PC-3 hybrids (five mice per hybrid) tumor growth was apparent in only one mouse, while tumors grew in all mice injected with the parental PC-3 cells. An *in vitro* assay showed that the Y chromosome did not suppress anchorage-independent growth of PC-3 cells. We found that addition of the Y chromosome suppressed tumor formation by PC-3 in athymic nude mice, and that this block of tumorigenesis was independent of the *in vitro* growth properties of the cells. This observation suggests the presence of a gene important for prostate tumorigenesis on the Y chromosome.

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in American men (Jemal et al., 2002). To identify the chromosomal regions affected in prostate cancer several different

tools like Giemsa-banding, flourescent *in situ* hybridization, comparative genomic hybridization, loss of heterozygozity and gene expression microarray analyses are commonly used. Cytogenetic studies have shown several chromosomal imbalances occurring in prostate cancer, including loss of chromosomal material from 1q (Latini et al., 2001), 5q, 6q, 7q, 8p, 10q, 13q, 16q, 17q, 18q, Xq (review Brothman et al., 1999) and Y (Konig et al., 1996). Earlier studies have shown that the Y chromosome is one of the most frequently lost chromosomes in prostate cancer. The Y chromosome was reported to be lost in 53% of 42 samples (Konig et al., 1994), 31% of 35 samples (Baretton et al., 1994) and 89% of 12 samples (Haapala et al., 2001) of prostate tumor examined. These studies on Y chromosome and other chromosomes lost in the prostate cancer imply loss of a gene whose loss of function results in cancer incidence or progression. Current evidence indicates that the Y chromosome is lost in several other cancers including leukemia (Sandberg, 1991), bladder cancer (Sauter et al., 1995), esophageal carcinoma (Hunter et al., 1993), gastric cancer (Castedo et al., 1992) and pancreatic cancer (Wallrapp et al., 2001). However, the significance of the loss of the Y chromosome in the development or progression of different types of cancer is still unknown. Most of the previous studies on the Y chromosome have focused on whole chromosome gain or loss in tumor tissue and hence failed to identify the minimal critical region involved in tumorigenesis. One study reported loss of the short arm of the Y chromosome in 35% of prostate tumor samples (Jordan et al., 2001). In a cohort study of four different ethnic groups, statistically significant association was observed between the Y chromosome and prostate cancer. Japanese men who were 65 years or younger carrying a particular Y chromosome lineage were found to be at 2.8 times higher risk for developing prostate cancer (Paracchini et al., 2003). A separate population study done on Japanese men showed that people with a

specific polymorphism at microsatellite locus DYS19 (Yp11.3) were predisposed to prostate cancer (Ewis et al., 2002). These recent reports and other published results support a definite role for the Y chromosome in maintaining the cellular integrity in the prostate. Because loss of the Y chromosome is common in prostate cancer cells and not in the normal stromal cells (van Dekken and Alers, 1993), we hypothesize that loss of the Y chromosome plays a significant role in the genesis/progression of prostate cancer.

MATERIALS AND METHODS

TAGGING Y CHROMOSOME

A Chinese hamster/human hybrid cell line containing the Y chromosome, GM06317 (Coriell Institute for Medical Research, New Jersey) was maintained in MEM Eagle's medium supplemented with 15% fetal calf serum. The cells were transfected with the *hisD* containing targeting vector pHTtkM3 (Farr et al., 1991) by electroporation. After transfection, the cells were grown in selective medium lacking histidine and containing 5mM histidinol (Gibco-BRL).

DETECTION OF THE Y CHROMOSOME

Fluorescence *in situ* hybridization: FISH was done as described (Padalecki et al., 2001). The probe for *hisD* was prepared as follows. A 3.2 Kb XhoI-EcoRI fragment containing *hisD* was biotin-labeled by nick translation (Gibco-BRL). Tyramide Signal Amplification (TSA)-FISH was done on Chinese hamster/human hybrids with a tagged Y chromosome following the procedure of Schriml et al., with minor modifications (Schriml et al., 1999). 4,6-diamidino-2-phenylindole was used as the counter stain. The slide was viewed using a Ziess Axioscop

fluorescence microscope and the image captured using Applied Imaging's Probe Vision. After washing and denaturing, the slide was reprobed with a Y chromosome probe (Vysis, Downers Grove, IL). PC-3 cells were probed with human Y chromosome paint.

MICROCELL MEDIATED CHROMOSOME TRANSFER (MMCT)

MMCT was done following the protocol described, with minor modifications (McNeill and Brown, 1980). Briefly, Chinese hamster/human hybrids with the tagged Y chromosome served as the donor and PC-3 as the recipient. The donor cells were treated with 0.06 μg/ml of colcemid for 24-48 hours. Microcells were obtained after centrifugation at 15,000 rpm for 30 minutes at 34°C in the presence of 10 μg/ml of cytochalasinB. Microcells were resuspended in 100 μg/ml of phytohemagglutinin-P (PHA) and later fused with PC-3 cells in the presence of 50% polyethylene glycol (PEG) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium. After 24-48 hours, the cells were put in selection medium DMEM/F-12 (without histidine) containing 5 mM histidinol. Following 10-14 days in culture, the resultant hybrids were tested for the presence of the Y chromosome. Control PC-3 cells were either untreated or transfected with the pHTtkM3 vector and selected in histidinol containing DMEM/F-12 medium.

ASSAYS FOR TUMOR SUPPRESSION

In vivo tumorigenicity assay

2×10^6 cells were injected subcutaneously into the shoulder of five weeks old male Balb/c *nu/nu* mice. Each cell line was injected into five animals. Tumor growth was measured twice weekly and tumor volume was calculated using the formula, (length x width^2)/2. Any tumor

formed in the experimental group was aseptically removed and expanded in DMEM/F-12 (nonselective) for further analysis. Those mice that did not form tumors were monitored for three months. Prior to sacrifice, these mice were anesthetized and whole body scan was done by exposing to X-ray at 35kV for 6s (Faxitron X-ray Corporation, Buffalo Grove, IL).

Anchorage-independent growth studies

PC-3 or PC-3 hybrids containing the Y chromosome were seeded on 60mm soft agar plates (n=4) at a density of 1000 cells/plate. The soft agar plates have a base layer containing 0.4% agarose, 10% DMEM and 10% fetal bovine serum and a top layer consisting of 0.24% agarose. The cells were fed after 1-1.5 weeks and scored after two weeks using p-iodonitrotetrazolium violet as the dye and counted using the software GelExpert (Nucleotech Corporation, San Mateo, CA). Doubling times for PC-3 and PC-3 hybrids were determined by plating 1 x 10^4 cells/well in 24-well plates and the cells were counted using hemacytometer for a period of time.

CHARACTERIZATION OF THE HYBRIDS

All hybrids injected were assayed for the presence of 35 Y chromosome specific markers. The three prostate cancer cell lines, DU145, LnCaP and PC-3, were also simultaneously tested for these markers. Each 20 μl polymerase chain reaction (PCR) consisted of 120 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl, pH8.3, 1.5–3.0 mM $MgCl_2$, 200 μM dNTPs, 10 ng of each primers and 0.15 U of Taq polymerase (Gene Choice, Frederick, MD). A stepdown program was used for amplification (Underhill et al., 1997). PCR products were visualized on 1.5% agarose gel by ethidium bromide staining.

Hybridization of BAC microarrays were performed to assay for chromosomal changes. Whole genome microarray analysis was done on 1-2 Mb human whole genome microarray slides (Spectral Genomics, Houston) using the manufacturer's protocol. Statistical analysis was done using the SpectralWare, Version 2.0 (Spectral Genomics, Houston).

RESULTS

Tagging and transferring of Y chromosome to PC-3 cells

We directly tested for the suppressive effect of the Y chromosome by first tagging the human Y chromosome with a selectable marker. A Chinese hamster/human cell hybrid, GM06317 was used as the source of the Y chromosome. We successfully targeted the histidinol (*hisD*) resistance gene to the *MIC2 locus* on Yp by homologous recombination using the vector pHTtkM3 (Fig. 1*a*). The presence of the *hisD* gene on the Y chromosome was detected by Tyramide Signal Amplification-Fluorescence *in situ* Hybridization (TSA-FISH) (Fig. 1*b*). The same metaphase was reprobed with a commercially available human chromosome paint specific for Y chromosome repetitive sequences. Several independent hamster/human cell hybrids with a tagged Y chromosome were established, and three independent lines, pHTtkM3 1-5B, pHTtkM3 2-2C and pHTtkM3 2-6B, were used in further experiments. Among the three prostate cancer cell lines tested, Y chromosome markers (Table1) were detected in DU145 and LnCaP (data not shown). As reported earlier (Bernardino et al., 1997), we also did not observe Y chromosome sequences in PC-3 cells by PCR analyses (Table 1) and by FISH (data not shown). The *hisD*-tagged Y chromosome was transferred to PC-3 cells using MMCT. Three independent chromosome transfer experiments were done for PC-3. From

each of these experiments, 25 clones were picked randomly and expanded. A total of twelve different PC-3 hybrids, each carrying a donor Y chromosome, were tested for tumorigenicity.

In vivo tumorigenicity assays

To assess whether the Y chromosome can revert the tumorigenic phenotype of PC-3, we tested the tumorigenicity of PC-3 hybrids in Balb/c *nu/nu* mice. Twelve PC-3 hybrids were injected subcutaneously into the shoulder using 2 x 106 cells per injection. Out of 60 mice injected, tumor growth was apparent in only one mouse. In contrast, all mice (n>5) injected with parental PC-3 cell line developed tumors (Fig. 2). An additional ten mice were injected with PC-3 cells that had been stably transfected with the targeting vector pHTtkM3 and selected in histidinol. These mice consistently formed tumors at a similar rate of incidence as untransfected PC-3 cells (data not shown). None of the mice exhibited any signs of metastasis as evident from X-ray scans (data not shown).

Anchorage-independent growth studies

In contrast to our *in vivo* results, the Y chromosome did not inhibit the anchorage-independent growth of PC-3 cells. Further, the presence of the Y chromosome had no effect on the doubling time of the cells which was less than 24 hours for both PC-3 cells and it's hybrids carrying Y chromosome (data not shown). In contrast to the *in vivo* results, where the Y chromosome was able to block tumorigenicity, all twelve PC-3 hybrids plated at 1000 cells/ 60 mm plate, formed colonies in soft agar (Fig. 3). Indeed, clones containing the Y chromosome formed more colonies in soft agar than the parental PC-3 cell line, suggesting a positive effect of the Y chromosome on cell growth *in vitro*. Subclones isolated from the soft

agar experiment were tested for their ability to form tumors *in vivo*. Only one of the nine subclones, 2-2 C12 E, consistently formed tumors (Fig. 4). A second clone, 2-2 C12 A, formed a tumor in only one mouse out of the five injected. The remaining seven clones (n=5 mice) did not form tumors. These data indicate that the Y chromosome does not inhibit the anchorage-independent growth of PC-3 cells, even though tumor growth is inhibited *in vivo*.

Characterization of PC-3 hybrids

Since one of the PC-3 hybrids, 2-6B E2, developed a tumor, we characterized the Y chromosome sequences present in the hybrid cell line by PCR and compared the results to those from the remaining PC-3 hybrids that did not form tumors. The hybrid 2-6B E2, retained all 35 markers we tested (Table 1). The PC-3 hybrids 2-2 C1, 2-2 C2 and 2-2 C3 had deletions on Yq (Table 1) but still suppressed tumor formation *in vivo* (Fig. 1*b*). The whole genome microarray analysis done comparing untransfected PC-3 cells to PC-3 hybrid, 2-2 C12 E that formed tumors in all five mice injected did not show any additional chromosomal copy number changes in the hybrid. Most of the subclones isolated from the soft agar exhibited various deletions (Table 1), especially the subclones of PC-3 hybrid 2-2 C1 that had major deletions on the long arm of the Y chromosome. These hybrids had retained only approximately 850 kb in the proximal Yq and had a single distal Yq marker, SHGC-7605 present. Despite this loss on the long arm, the tumorigenicity of PC-3 was suppressed. We conclude that these deleted regions are not critical for the tumor suppression potential of the Y chromosome. Thus, we have narrowed down the region that harbors tumor suppression activity primarily to the short arm.

DISCUSSION

One unique feature of prostate cancer is its multifocality. Several independent genetically heterogeneous lesions can be found both within a tumor and also between tumors (Qian et al., 1995). The exact sequence of genetic events occurring during the progression of prostate cancer is not well understood. Though loss of the Y chromosome is a common numerical aberration observed in prostate cancer, the significance of this loss has not yet been examined. Here, we have developed a model to test functionally the role of the Y chromosome in prostate cancer tumorigenicity. Of the three human prostate cancer cell lines, PC-3, DU145 and LnCaP, only PC-3 was found to be completely devoid of Y chromosome sequences. This is in agreement with previous reports on PC-3 where cytogenetic analyses have shown loss of the Y chromosome in this cell line (Bernardino et al., 1997). Therefore, we chose PC-3 to study the effect of the addition of the Y chromosome on the tumorigenic phenotype of the prostate cancer cells. As evident from our data, addition of the Y chromosome suppressed the tumorigenicity of the parental PC-3 cells. The observation that tumor suppression was seen in 59 out of 60 mice challenged with PC-3 hybrids strongly supports the presence of a tumor suppressor gene on the Y chromosome. In a previous study using a similar approach, chromosome 10 was shown to suppress the tumorigenicity of the PC-3 cell line (Sanchez et al., 1996). The introduction of chromosome 10 into PC-3 cells restored an apoptotic pathway that is absent in the parental cell line. Addition of human chromosome 5 to PC-3 cells suppressed tumorigenicity and changes were noted in signaling mediated through α-catenin and E-cadherin (Ewing et al., 1995). Although the exact mechanisms of action are not known, human chromosomes 12 (Berube et al., 1994) and 17q

(Murakami et al., 1995) have also been shown to suppress tumor formation by the prostate cancer cell line PPC-1, a derivative of PC-3. The addition of an intact human chromosome 18 to PC-3 cells could only reduce the tumor growth rate of PC-3 hybrids when injected into nude mice (Padalecki et al., 2003). The insertion of chromosomes 2, 7, 8, 10, 11, 12 and 16 did not reduce the tumorigenicity in the Dunning rat prostate cancer model (Ichikawa et al., 2000), and chromosome 3 failed to suppress tumor formation by DU145 (Berube et al., 1994). The suppression of tumor growth observed after introduction of the Y chromosome into PC-3 cells is most likely due to the presence of the transferred Y chromosome and not due to a random effect since not all the chromosomes have the ability to suppress the tumorigenicity of the prostate cancer cells. The histidinol selection of PC-3 hybrids did not interfere with the tumorigenic potential of the cells as parental PC-3 cells transfected with the vector pHTtkM3 and selected in histidinol consistently formed tumors *in vivo*.

The fact that the introduction of the Y chromosome into PC-3 did not reduce colony formation in soft agar suggests that different genetic mechanisms are involved in regulating *in vivo* and anchorage-independent growth of the PC-3 hybrids. Lack of correlation of the anchorage-independent phenotype with the tumorigenic phenotype of cancer cells has been reported by others (Goyette et al., 1992; Murakami et al., 1995). In the soft agar assay, the efficiency of colony formation by the parental cell line PC-3 (1.3%), we observed, is comparable to what other investigators have reported for the cell line (Srikantan et al., 2002). At this time, the reason why PC-3 hybrids 2-2 C12 E and 2-2 C12 A formed tumors in nude mice after subculturing in soft agar is not known. One possibility is the presence of microdeletions on the chromosome as only a sampling of the chromosome is determined by

PCR analysis. The whole genome microarray analysis did not show any new changes in other chromosomes in the hybrid 2-2 C12E. Alternatively, a gene important for preventing tumor growth *in vivo* may have been inactivated by point mutation in these two cell lines. A more intensive approach to detect these deletions in the hybrids is in progress.

It is not surprising to see a drastic reversal in the tumorigenic phenotype of a cancer cell having multiple genetic changes just by replacing one/portion of an affected chromosome (Goyette et al., 1992). PC-3 contains a multitude of genetic aberrations (Aurich-Costa et al., 2001) including mutated p53 (Isaacs et al., 1991). The mechanism of Y chromosome tumor suppression in PC-3 cells is at present unknown. The Y chromosome contains many genes whose functions have not been closely examined in the context of cancer. It is also worth noting that in a deletion analysis on prostate cancer samples, loss of six genes lying between Yp11.3 and Yq12.1 (Perinchery et al., 2000) was observed. In our study, all hybrids retained the short arm of the Y chromosome. Furthermore, a study by Jordan et al. showed that loss of Yp is more frequent than Yq in prostate tumor samples (Jordan et al., 2001). However, the same group noticed normal copy number for the Y chromosome using touch preparation of tumor samples instead of paraffin-embedded sections (Tricoli, 1999). Our *in vivo* data support the presence of a tumor suppressor gene on the Y chromosome. A gene expression analysis provided clues that expression of certain Y chromosome specific genes including SRY and ZFY on the short arm are altered in prostate tumors (Dasari et al., 2001).

These published observations, taken together with our findings on the suppression of tumorigenicity by the Y chromosome, strongly suggest the presence of a gene on the Y

chromosome that is involved in the development of prostate cancer. Further analysis of the hybrid cell lines we described will facilitate the identification of the gene(s) responsible for the suppression of PC-3 tumorigenesis and allow us to determine the mechanism of this suppression. Currently, we are focusing on identifying the minimal region on the short arm of Y chromosome that has the tumor suppression property.

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REFERENCES

Aurich-Costa J, Vannier A, Gregoire E, Nowak F, and Cherif D. 2001. IPM-FISH, a new M-FISH approach using IRS-PCR painting probes: application to the analysis of seven human prostate cell lines. Genes Chromosomes Cancer 30:143-160.

Baretton GB, Valina C, Vogt T, Schneiderbanger K, Diebold J, and Lohrs U. 1994. Interphase cytogenetic analysis of prostatic carcinomas by use of nonisotopic in situ hybridization. Cancer Res 54:4472-4480.

Bernardino J, Bourgeois CA, Muleris M, Dutrillaux AM, Malfoy B, and Dutrillaux B. 1997. Characterization of chromosome changes in two human prostatic carcinoma cell lines (PC-3

and DU145) using chromosome painting and comparative genomic hybridization. Cancer Genet Cytogenet 96:123-128.

Berube NG, Speevak MD, and Chevrette M. 1994. Suppression of tumorigenicity of human prostate cancer cells by introduction of human chromosome del(12)(q13). Cancer Res 54:3077-3081.

Brothman AR, Maxwell TM, Cui J, Deubler DA, and Zhu XL. 1999. Chromosomal clues to the development of prostate tumors. Prostate 38:303-312.

Castedo S, Correia C, Gomes P, Seruca R, Soares P, Carneiro F, and Sobrinho-Simoes M. 1992. Loss of Y chromosome in gastric carcinoma. Fact or artifact? Cancer Genet Cytogenet 61:39-41.

Dasari VK, Goharderakhshan RZ, Perinchery G, Li LC, Tanaka Y, Alonzo J, and Dahiya R. 2001. Expression analysis of Y chromosome genes in human prostate cancer. J Urol 165:1335-1341.

Ewing CM, Ru N, Morton RA, Robinson JC, Wheelock MJ, Johnson KR, Barrett JC, and Isaacs WB. 1995. Chromosome 5 suppresses tumorigenicity of PC3 prostate cancer cells: correlation with re-expression of alpha-catenin and restoration of E-cadherin function. Cancer Res 55:4813-4817.

Ewis AA, Lee J, Naroda T, Sasahara K, Sano T, Kagawa S, Iwamoto T, and Nakahori Y. 2002. Linkage between prostate cancer incidence and different alleles of the human Y-linked tetranucleotide polymorphism DYS19. J Med Invest 49:56-60.Farr C, Fantes J, Goodfellow P, and Cooke H. 1991. Functional reintroduction of human telomeres into mammalian cells. Proc Natl Acad Sci U S A 88:7006-7010.

Goyette MC, Cho K, Fasching CL, Levy DB, Kinzler KW, Paraskeva C, Vogelstein B, and Stanbridge EJ. 1992. Progression of colorectal cancer is associated with multiple tumor suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect via chromosome transfer. Mol Cell Biol 12:1387-1395.

Haapala K, Rokman A, Palmberg C, Hyytinen ER, Laurila M, Tammela TL, and Koivisto PA. 2001. Chromosomal changes in locally recurrent, hormone-refractory prostate carcinomas by karyotyping and comparative genomic hybridization. Cancer Genet Cytogenet 131:74-78.

Hunter S, Gramlich T, Abbott K, and Varma V. 1993. Y chromosome loss in esophageal carcinoma: an in situ hybridization study. Genes Chromosomes Cancer 8:172-177.

Ichikawa T, Hosoki S, Suzuki H, Akakura K, Igarashi T, Furuya Y, Oshimura M, Rinker-Schaeffer CW, Nihei N, Barrett JC, Isaacs JT, and Ito H. 2000. Mapping of metastasis suppressor genes for prostate cancer by microcell-mediated chromosome transfer. Asian J Androl 2:167-171.

Isaacs WB, Carter BS, and Ewing CM. 1991. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. Cancer Res 51:4716-4720.

Jemal A, Thomas A, Murray T, and Thun M. 2002. Cancer statistics, 2002. CA Cancer J Clin 52:23-47.

Jordan JJ, Hanlon AL, Al Saleem TI, Greenberg RE, and Tricoli JV. 2001. Loss of the short arm of the Y chromosome in human prostate carcinoma. Cancer Genet Cytogenet 124:122-126.

Konig JJ, Teubel W, Romijn JC, Schroder FH, and Hagemeijer A. 1996. Gain and loss of chromosomes 1, 7, 8, 10, 18, and Y in 46 prostate cancers. Hum Pathol 27:720-727.

Konig JJ, Teubel W, van Dongen JW, Romijn JC, Hagemeijer A, and Schroder FH. 1994. Loss and gain of chromosomes 1, 18, and Y in prostate cancer. Prostate 25:281-291.

Latini JM, Rieger-Christ KM, Wang DS, Silverman ML, Libertino JA, and Summerhayes IC. 2001. Loss of heterozygosity and microsatellite instability at chromosomal sites 1q and 10q in morphologically distinct regions of late stage prostate lesions. J Urol 166:1931-1936.

McNeill CA and Brown RL. 1980. Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells. Proc Natl Acad Sci U S A 77:5394-5398.

Murakami YS, Brothman AR, Leach RJ, and White RL. 1995. Suppression of malignant phenotype in a human prostate cancer cell line by fragments of normal chromosomal region 17q. Cancer Res 55:3389-3394.

Padalecki SS, Weldon KS, Reveles XT, Buller CL, Grubbs B, Cui Y, Yin JJ, Hall DC, Hummer BT, Weissman BE, Dallas M, Guise TA, Leach RJ, and Johnson-Pais TL. 2003. Chromosome 18 suppresses prostate cancer metastases. Urol Oncol 21:366-373.

Padalecki SS, Johnson-Pais TL, Killary AM, and Leach RJ. 2001. Chromosome 18 suppresses the tumorigenicity of prostate cancer cells. Genes Chromosomes Cancer 30:221-229.

Paracchini S, Pearce CL, Kolonel LN, Altshuler D, Henderson BE, and Tyler-Smith C. 2003. A Y chromosomal influence on prostate cancer risk: the multi-ethnic cohort study. J Med Genet 40:815-819.

Perinchery G, Sasaki M, Angan A, Kumar V, Carroll P, and Dahiya R. 2000. Deletion of Y-chromosome specific genes in human prostate cancer. J Urol 163:1339-1342.

Qian J, Bostwick DG, Takahashi S, Borell TJ, Herath JF, Lieber MM, and Jenkins RB. 1995. Chromosomal anomalies in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence in situ hybridization. Cancer Res 55:5408-5414.

Sanchez Y, Lovell M, Marin MC, Wong PE, Wolf-Ledbetter ME, McDonnell TJ, and Killary AM. 1996. Tumor suppression and apoptosis of human prostate carcinoma mediated by a genetic locus within human chromosome 10pter-q11. Proc Natl Acad Sci U S A 93:2551-2556.

Sandberg AA. 1991. Chromosome abnormalities in human cancer and leukemia. Mutat Res 247:231-240.

Sauter G, Moch H, Wagner U, Novotna H, Gasser TC, Mattarelli G, Mihatsch MJ, and Waldman FM. 1995. Y chromosome loss detected by FISH in bladder cancer. Cancer Genet Cytogenet 82:163-169.

Schriml LM, Padilla-Nash HM, Coleman A, Moen P, Nash WG, Menninger J, Jones G, Ried T, and Dean M. 1999. Tyramide signal amplification (TSA)-FISH applied to mapping PCR-labeled probes less than 1 kb in size. Biotechniques 27:608-613.

Srikantan V, Valladares M, Rhim JS, Moul JW, and Srivastava S. 2002. HEPSIN inhibits cell growth/invasion in prostate cancer cells. Cancer Res 62:6812-6816.

Tricoli JV. 1999. Y chromosome enumeration in touch preparations from 42 prostate tumors by interphase fluorescence in situ hybridization analysis. Cancer Genet Cytogenet 111:1-6.

Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, Davis RW, Cavalli-Sforza LL, and Oefner PJ. 1997. Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 7:996-1005.

van Dekken H and Alers J. 1993. Loss of chromosome Y in prostatic cancer cells but not in stromal tissue. Cancer Genet Cytogenet 66:131-132.

Table 1. PC-3 hybrids exhibit regional losses on the Y chromosome

MARKERS	Distance between markers	PC-3 HYBRIDS													SOFT AGAR SUBCLONES									
		PC-3	1-5 BB	1-5 BE	1-5 BK	2-2 C1	2-2 C2	2-2 C3	2-2 C12	2-6 B A3	2-6 B B2	2-6 B C1	2-6 B D4	2-6 B E2	2-2 C1 A	2-2 C1 B	2-2 C1 C	2-2 C1 D	2-2 C1 E	2-2 C1 F	2-2 C12 A	2-2 C12 C	2-2 C12 E
SRY (Yp)	2562KB+	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DYS 252	259 KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	■	●	●
DYS 253	1222KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●
SHGC101838	2025KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DYS 266	703KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DYS 261	616KB	○	●	●	●	●	●	●	■	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DYS 260	161KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	■	■	●	●	●	●	●	●
DYS 288	75KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	○	●	●
DYS 257	961KB	○	●	○	○	●	○	■	■	●	●	●	●	●	●	●	●	●	●	●	●	■	●	●
CENTROMERE																								
DYS 271	307KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DYS 274	541KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DYS 276	966KB	○	●	●	●	●	●	■	●	●	●	●	●	●	●	○	○	○	○	○	●	●	●	●
DYS 277	183KB	○	●	●	●	●	●	●	●	●	●	●	■	●	○	○	○	○	○	○	●	●	●	●
DYS 278	80KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	■	■	●	●
DYS 280	677KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
DYS 390	668KB	○	●	●	●	●	○	○	●	●	●	●	●	●	○	○	○	○	○	○	●	●	○	●
DYS 282	295KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
SHGC60455	560KB	○	●	●	●	○	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
DYS 198	441KB	○	●	●	■	●	●	●	●	■	■	●	●	■	○	○	○	○	○	○	●	■	●	●
DYS 289	250KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	■	■	●	●
DYS 200	182KB	○	●	●	●	●	●	●	●	●	●	●	■	●	○	○	○	○	○	○	●	●	●	●
DYS 199	92KB	○	●	●	●	○	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
DYS 201	260KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
DYS 202	75KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
DYS 213	1861KB	○	●	●	●	○	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
DYS 215	793KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	●	●	●
DYS 219	641KB	○	●	●	●	■	●	●	●	●	●	●	■	●	○	○	○	○	○	○	●	●	●	●
DYS 221	416KB	○	●	●	■	○	■	○	●	●	●	○	●	●	○	○	○	○	○	○	●	○	●	●
SHGC9460	785KB	○	●	●	●	○	●	●	●	●	●	●	●	●	○	○	○	○	○	○	●	●	●	●
DYS 230	549KB	○	●	●	●	○	○	○	●	●	●	●	■	●	○	○	○	○	○	○	●	●	●	●
DYS 379	168KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	■	○	○	○	○	●	●	●	●
SHGC7605	7KB	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DYS 378	ND	○	●	●	●	○	●	○	●	●	■	●	●	●	○	○	○	○	○	○	○	●	■	●
DYS 247	1262KB	○	●	●	●	■	■	■	●	■	■	●	●	■	○	○	○	○	○	○	○	●	●	●
DYS 241(Yq)	2561KB	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	●	●	●

● Marker present ■ Marker was amplified to a lesser extent than the control DNA

○ Marker absent

All markers are listed according to their cytogenetic position on the chromosome. The distance between each marker is based on UCSC genome browser, July 2003.

[+] Distance from the telomere. ND, not determined.

Figure Legends

Figure 1. Tagging of human Y chromosome. **A**) Y chromosome in hamster/human hybrid cell line was tagged with the bacterial gene histidinol dehydrogenase, *hisD*, using the vector pHTtkm3. The marker *hisD* was targeted to *MIC2* locus on the short arm of Y chromosome. SV2his has SV40 ori, *hisD* and SV40 IVS/polyA sequences. **B**) Chinese hamster/human hybrid cell showing the marker *hisD* (green) targeted to Y chromosome. Inset shows fluorescence *in situ* hybridization done subsequently using a Y chromosome paint (pink) on the same metaphase.

Figure 2. Human Y chromosome suppresses the tumor growth of PC-3 in nude mice. **A**) Out of 25 mice injected with PC-3 hybrids, only one mouse injected with 2-6 B E2 (n=5) formed tumor, while all five mice injected with PC-3 cells grew tumors. **B**) Suppression of tumor growth by seven independent PC-3 hybrid clones (n=5). All five mice injected with PC-3 cells grew tumors. *Growth curve for one mouse that grew a tumor out of five injected with 2-6 B E2. Bar indicates the standard error.

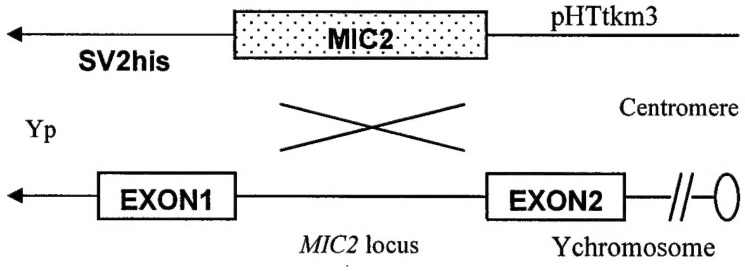
Figure 3. Presence of Y chromosome did not suppress the anchorage-independent growth of PC-3. All PC-3 hybrids, plated 1000 cells/ dish grew well on soft agar. Bar indicates the standard error.

Figure 4. Soft agar subcultured PC-3 hybrids still maintained the non-tumorigenic phenotype. Only two subclones isolated from soft agar formed tumor *in vivo*. All five mice injected with 2-2 C12 E grew tumor. A tumor grew in only one mouse (n=5) injected with 2-2 C12 A. Tumor

growth was not observed in mice (n=5) injected with rest of the seven subclones. * Growth curve for one mouse out of five injected with 2-2 C12A. Bar indicates the standard error.

Figures

A



B

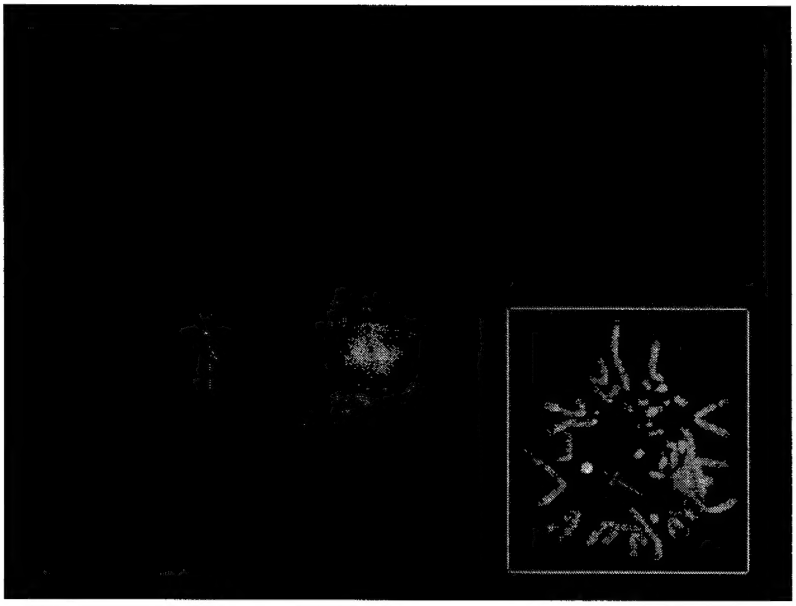
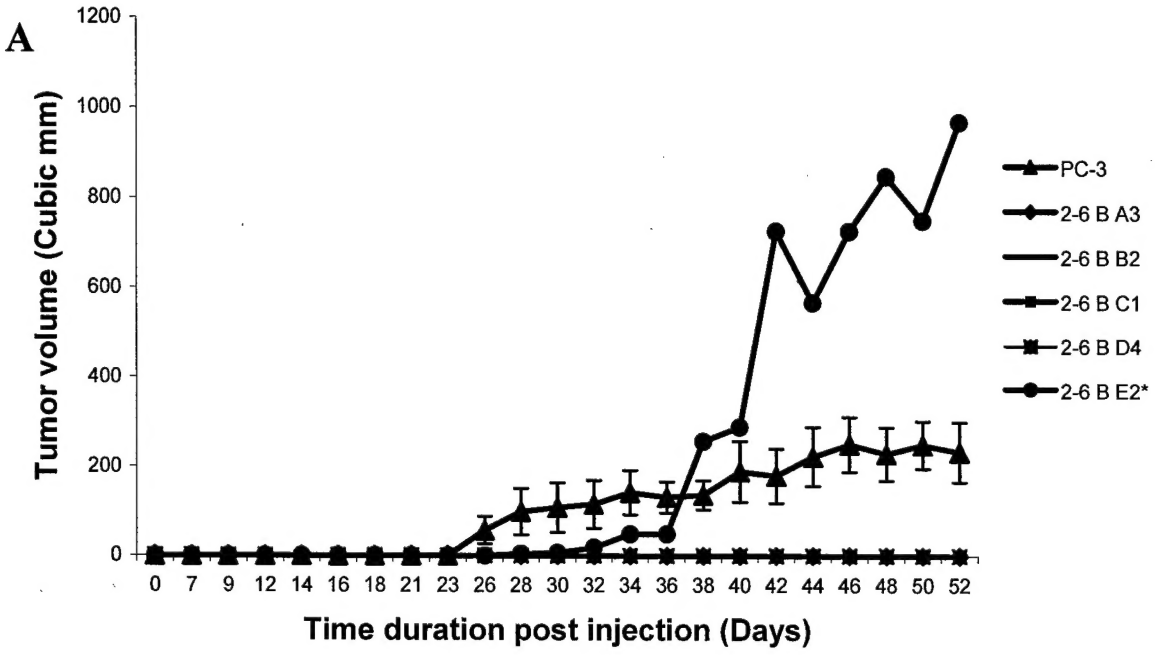
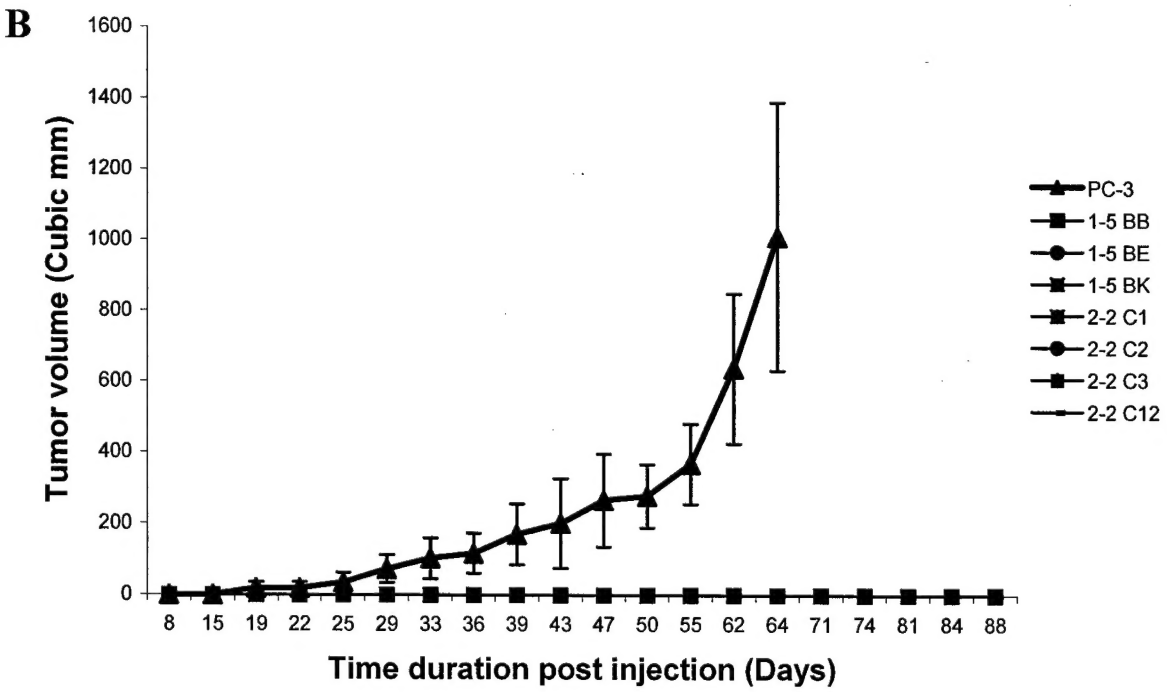


Figure 1

Figure 2


A
Tumor volume (Cubic mm)
1200
1000
800
600
400
200
0
0 7 9 12 14 16 18 21 23 26 28 30 32 34 36 38 40 42 44 46 48 50 52
Time duration post injection (Days)
PC-3
2-6 B A3
2-6 B B2
2-6 B C1
2-6 B D4
2-6 B E2*


B
Tumor volume (Cubic mm)
1600
1400
1200
1000
800
600
400
200
0
8 15 19 22 25 29 33 36 39 43 47 50 55 62 64 71 74 81 84 88
Time duration post injection (Days)
PC-3
1-5 BB
1-5 BE
1-5 BK
2-2 C1
2-2 C2
2-2 C3
2-2 C12

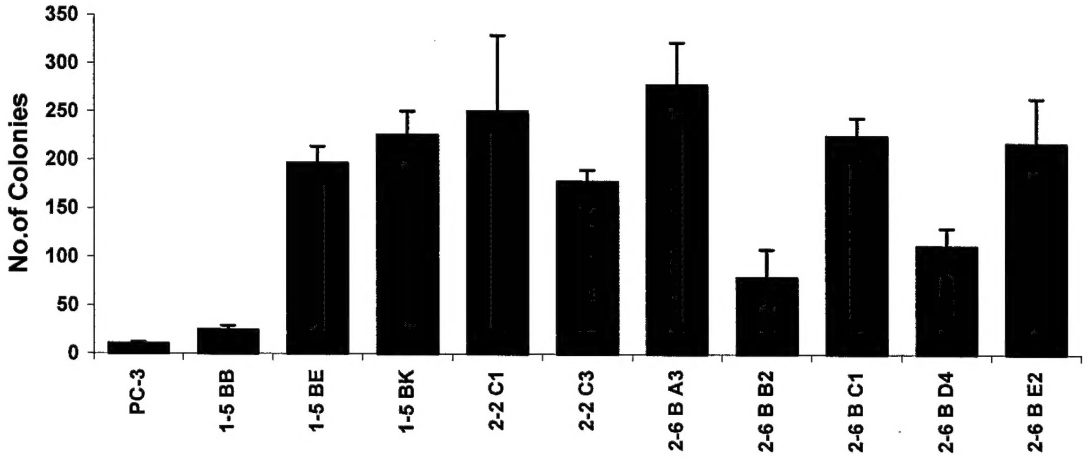

No.of Colonies
350
300
250
200
150
100
50
0
PC-3
1-5 BB
1-5 BE
1-5 BK
2-2 C1
2-2 C3
2-6 B A3
2-6 B B2
2-6 B C1
2-6 B D4
2-6 B E2

Figure 3

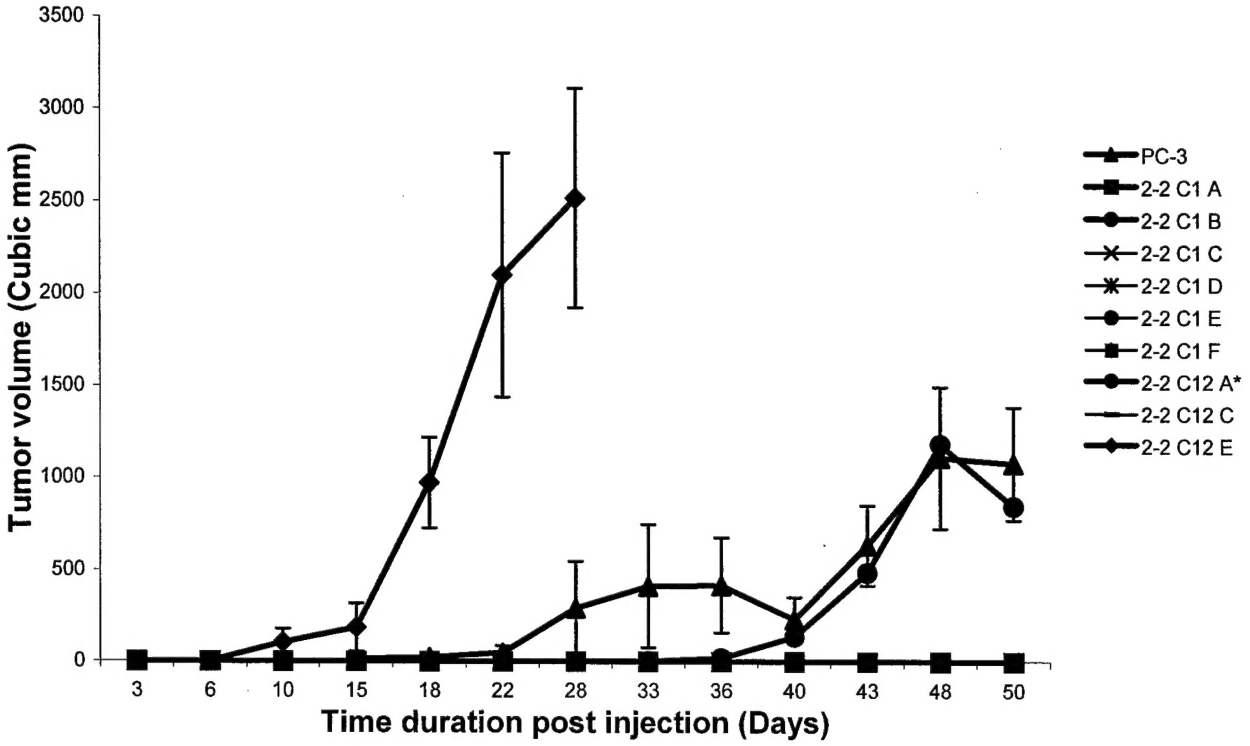

3500
3000
2500
2000
1500
1000
500
0
Tumor volume (Cubic mm)
3
6
10
15
18
22
28
33
36
40
43
48
50
Time duration post injection (Days)
PC-3
2-2 C1 A
2-2 C1 B
2-2 C1 C
2-2 C1 D
2-2 C1 E
2-2 C1 F
2-2 C12 A*
2-2 C12 C
2-2 C12 E

Figure 4